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# A Test for Genetic Variation for Resistance to Effects of Seawater Acidification on the Skeletal Development of Sea Urchin Larvae

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**A Test for Genetic Variation for Resistance to Effects of Seawater Acidification on  
the Skeletal Development of Sea Urchin Larvae**

by

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8/6/14

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## Abstract

Rising levels of atmospheric CO<sub>2</sub> are altering global ocean chemistry, including a decline in the pH of surface waters. Under more acidified conditions, marine organisms that build shells and skeletons are under increasing risk of a reduction in their ability to deposit calcium carbonate. Relatively little is known about genetic variation in the capacity of organisms to respond to such effects. We examined skeletal growth in larval sea urchins to examine genetic variation in their sensitivity to elevated CO<sub>2</sub> under exposure at two life history stages: at fertilization, and during larval development. Using gametes of the purple-spined sea urchin (*Arbacia punctulata*), we carried out single-pair crosses in blocks of 3 males x 3 females for a total of 9 sibships, repeated over 7 blocks. Fertilizations were done in seawater saturated at either current (392 ppm) or 2.5x-current (980 ppm) CO<sub>2</sub>, and the resulting embryos from each cross were reared over 3 days to four-arm larvae under each of the same two CO<sub>2</sub> conditions. Nine landmarks on larvae were used to calculate both skeletal and soft body measurements. Exposure to elevated CO<sub>2</sub> during larval development significantly reduced the length of the postoral arms and body rods and increased postoral arm asymmetry. Surprisingly, exposure to elevated CO<sub>2</sub> during fertilization also reduced the subsequent growth of anterolateral arms, body rods, and body length. We found significant additive and non-additive genetic variation for growth of certain characters but no evidence of genetic variation for the effects of elevated CO<sub>2</sub> on growth. These results suggest that this population may not have the genetic capacity for an evolutionary response to elevated CO<sub>2</sub> under predicted near-future conditions.

## **Table of Contents:**

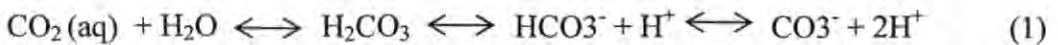
I.	Introduction.....	1
II.	Methods	
i.	Collection and Handling of Study Organisms.....	3
ii.	Spawning and Fertilization.....	4
iii.	Larval Rearing and Sampling.....	5
iv.	Larval Measurements.....	6
III.	Results	
i.	Rearing Effects of CO <sub>2</sub> on Larval Development.....	8
ii.	Fertilization Effect of CO <sub>2</sub> on Larval Development.....	8
iii.	Effects of CO <sub>2</sub> on the Coefficient of Variation.....	9
iv.	Genetic Variation for Resistance to CO <sub>2</sub> .....	11
IV.	Discussion.....	12
V.	References.....	15

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## Introduction:

Carbon emissions, over the last century, have led to rising levels of atmospheric CO<sub>2</sub> causing a decline in ocean pH, a phenomenon known as ocean acidification. The ocean, which acts as a carbon sink, has absorbed approximately 30% of anthropogenic carbon emissions since 1980. Within the new few thousand years the ocean could absorb as much as 90% of carbon emissions (Sabine et al. 2004). Carbon dioxide dissolved in sea water undergoes a series of reactions that produce hydrogen ions, thereby reducing the pH of the ocean (Orr et al. 2005):



As a result of this process, surface ocean pH is estimated to be 0.1 units below preindustrial levels, and could decrease as much as 0.3-0.4 units within the next 90 years (Haugan et al. 1996, Fabry et al. 2008).

The decline in ocean pH can be especially detrimental to calcifying marine organisms because it alters the saturation state of calcium carbonate (CaCO<sub>3</sub>), a common component of marine skeletons (Feely et al. 2004). This process occurs because the presence of excess hydrogen ions leads to their binding to carbonate ions, producing bicarbonate. An increase in hydrogen will therefore reduce the concentration of carbonate and the ability of CaCO<sub>3</sub> to form. This process can lower growth rate and weaken skeletons of numerous calcifying organisms including bryozoans, mollusks, corals, and echinoderms (Orr et al. 2005, Raven et al. 2005).

The challenge of sequestering enough  $\text{CaCO}_3$  to form calcified skeletons can be of vital importance to the organisms that include a calcifying larval stage. Sea urchin larvae in particular serve as an excellent model for studies of ocean acidification because their development has been extensively studied and because their skeleton may be more sensitive to pH than those of other larval organisms (Beniash et al. 1997). Sea urchins even have a unique amorphous calcium carbonate material that makes up their skeleton during a part of their development, which is more soluble than the calcite of the adult skeleton (Politi et al. 2004). Under experimentally reduced pH, sea urchin larvae have shown a significantly shorter total body length, shorter lengths of particular skeletal elements and an increase in malformation of body structure (Clark et al. 2009, Brennard et al. 2010, Kurihara et al. 2004). A more fragile larval skeleton, as seen in these experiments, could lead to population declines because the survival of larvae influences recruitment success (Przeslawski et al. 2008).

Given the potential for an ecological shift from, calcifying to non-calcifying organisms, declines in sea urchin populations could lead to cascading effects in marine food webs and ecosystems (Clark et al. 2009). Due to the potential for an ecological effect, it is important to understand the potential for populations to adapt over time to ocean acidification. Evolutionary adaptation of populations can only occur given sufficient genetic variation. With greater genetic variation, comes the greater likelihood that populations will have genes that confer greater resistance to acidified conditions. Evidence supports the existence of among-species and among-population (e.g. from different geographical regions; Clark et al. 2009) variation in resistance to the effects of ocean acidification. However, there has been relatively little in species with calcifying

larvae of within-population genetic variation in resistant to acidification. For example, coccolithophore *Emiliana huxleyi*, a calcifying species with a short generation time, has been seen to adapt to higher CO<sub>2</sub> concentrations over several generations in lab experiments (Lohbeck et al. 2012), suggesting significant genetic variation within the population.

We carried out controlled crosses of sea urchins from a single population to examine the level of genetic variation for resistance to acidification in the growth of larvae. In particular our study addressed three questions about the growth of larval skeletons (1) Does exposure to increased CO<sub>2</sub> during development affect larval growth? (2) Does exposure to increased CO<sub>2</sub> during fertilization affect subsequent larval growth? (3) Do adults show genetic variation for larval growth or the response of larval growth to increased CO<sub>2</sub>? This information will help us understand how the structure of genetic variation within a calcifying species can play a role in the evolution of resistance to the future effects of ocean acidification.

## Materials and Methods

### *Collection and Handling of Study Organism*

To carry out crosses and larval rearing the laboratory, we used the purple spined sea urchin, *Arbacia punctulata*. Adults were collected from a rock groin on Sullivan's Island near Charleston, South Carolina, and maintained close to collection temperatures (approx. 24°C) at the Grice Marine Laboratory. Animals were maintained at approximately a 30-35ppt salinity and  $\leq 0.50$  ppm ammonium



level, with tank cleaning and water changes biweekly. Specimens were fed ad libitum a mixture of algae (*Ulva* sp. and *Gracilaria vermiculiforma*) collected locally and organic carrots.

### *Spawning and Fertilization*

Sea urchins were induced to spawn either through a low electrical voltage applied across the gonopores or through intracoelomic-injection of about 1 ml of 0.53 M KCl. Eggs were spawned directly into filtered sea water (FSW, taken from Hollings Marine Laboratory, Ft. Johnson) and washed twice before use. Dry sperm were collected into a micropipettes at a concentration of approximately  $4 \times 10^{10}$  sperm/ml. For experiments sperm was diluted to end concentrations of  $10^{4.3}$  /ml<sup>-1</sup> for the first run,  $10^{5.3}$  /ml<sup>-1</sup> for the second run, and  $10^{5.5}$  /ml<sup>-1</sup> for run three through six. Gametes were collected and diluted separately for each male and female.

Fertilizations were carried out in 13 x 100 mm culture tubes at room temperature (approx. 24°C). For each block, fertilizations were carried out in all pair-wise combinations among 3 males and 3 females, for a total of 9 sibships. The gametes of each pair were fertilized under two conditions for a total of 18 fertilization tubes: current level CO<sub>2</sub> levels (392 ppm), generated by bubbling FSW with hydrated air, and 2.5x current levels (980 ppm) generated by bubbling FSW with a hydrated mixture of three compressed gases (N<sub>2</sub>, O<sub>2</sub>, and 1%CO<sub>2</sub>/99% N<sub>2</sub>) using mass flow controllers (Aalborg; Orangeburg, NewYork). Approximately 600 eggs and 0.525 ml of sperm were added to the tubes for a



final volume of 5.247 ml. This process was repeated twice to generate two tubes for each sibship and CO<sub>2</sub> concentration. Eggs were allowed to settle in tubes for 45 min before sperm water was removed. Eggs were then washed into 1 l jars containing either 1x or 2.5x – current CO<sub>2</sub> FSW, so that fertilization conditions (1x and 2.5x CO<sub>2</sub>) were factorially crossed with rearing conditions (1x and 2.5x CO<sub>2</sub>). In total 36 jars were used (2 fertilization conditions x 2 rearing conditions x 3 sires x 3 dams) were arranged randomly on a table. FSW used for fertilization and rearing conditions had been bubbled with the appropriate gas for 1.5 to 2 h and allowed to equilibrate for at least 12 hours before each experimental run.

### *Larval Rearing and Sampling*

A stirring rack was used to continuously suspend larvae in culture jars. The stirring rack was built using a four-legged stand from PVC stand (Strathmann, 1987). A lower rack, suspended from the larger stand by coated wire, was utilized to suspend paddles into each jar. The lower rack and corresponding paddles were driven back and forth, by a small motorized arm connected to the end of the large stand, at 10 strokes min<sup>-1</sup>. The lid of each jar had a small hole large enough for the paddle shaft, so that the fulcrum for the paddle movement was the jar lid. A small tube constructed constructed from intramedic tubing (Inner Diameter: 0.76mm, Outer Diameter: 1.22mm) delivered a flow of gas to fill the headspace above the FSW. The air supply tubes delivered gas continuously throughout each run of the experiment in order to maintain the proper concentration of dissolved CO<sub>2</sub> in each jar, by Henry's Law. Sea urchin

larvae were allowed to develop approximately 72 hours, at which point the anterolateral (2<sup>nd</sup> pair of) arms had begun to form.

Larvae were collected by pouring the contents of each jar into a small cup with a 30- $\mu$ m mesh bottom. The filter cup was placed into a shallower dish to keep the larvae suspended in water above the filter. About 30 larvae per jar were collected into 2 ml microcentrifuge tubes in 1 ml seawater and 300  $\mu$ l of methanol was added. Larvae were kept at -20°C until measured within a few days.

### *Larval Measurements*

We measured the size of approximately 15 larvae from each tube by recording x, y, and z coordinates. Nine landmarks (Figure 1): the distal tips of each postoral and anterolateral arm, the nodes at which the arms met the body rods, the posterior point where the body rods met, and the medial positions of the two tissue bridges that connected the postoral arms and anterolateral arms, respectively. These coordinates were used to measure 7 features: the lengths of the left and right postoral and anterolateral arms; the lengths of the left and right body rod; body width; and two body length measurements (Fig. 2a and 2b).

Coordinates were collected by viewing the larva on a compound A microscope (CX31, Olympus) fitted with a camera lucida. A digitizing pad was viewed through the camera lucida while each landmark was brought into focus, and the x, y, and z coordinates were recorded using the digitizing pad (HP 12000U, Aiptek) coupled to a rotary encoder that recorded changes in the plane of focus. The data were recorded using the Landmark plug-in for Image J (National

Institutes of Health; US Digital). The coordinates were then brought on to an Excel Spreadsheet and used to calculate the lengths of body parts.

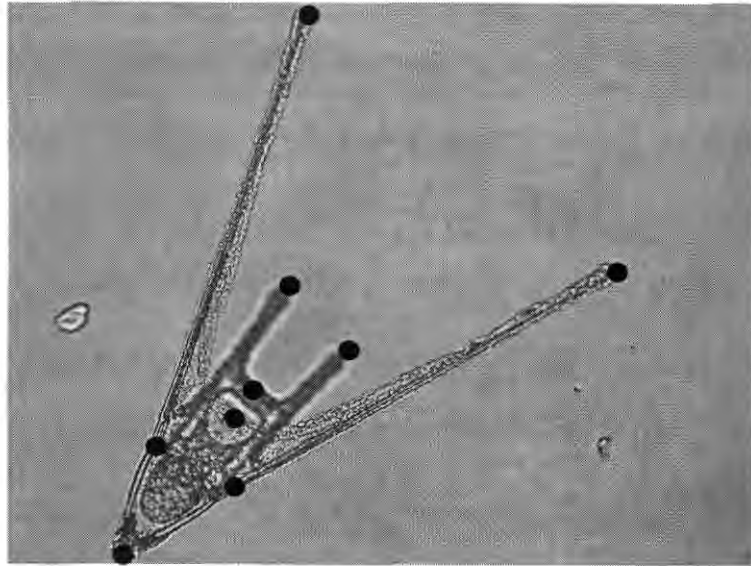


Figure 1. Landmarks used for measurement on *A. punctulata* larva. the distal tips of each postoral and anterolateral arm, the nodes at which the arms met the body rods, the posterior point where the body rods met, and the medial positions of the two tissue bridges that connected the postoral arms and anterolateral arms, respectively

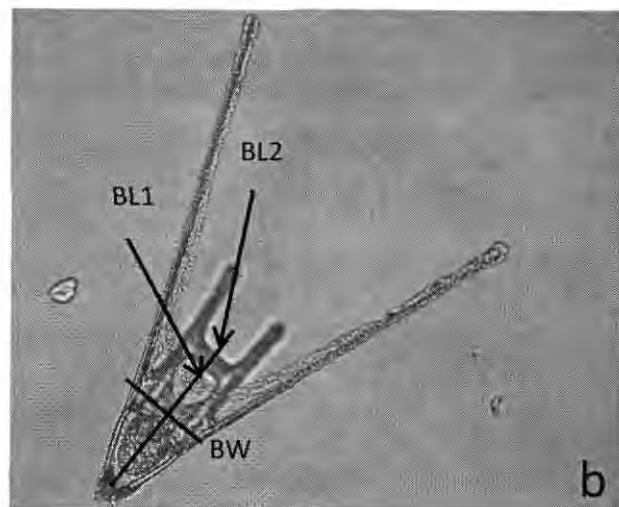
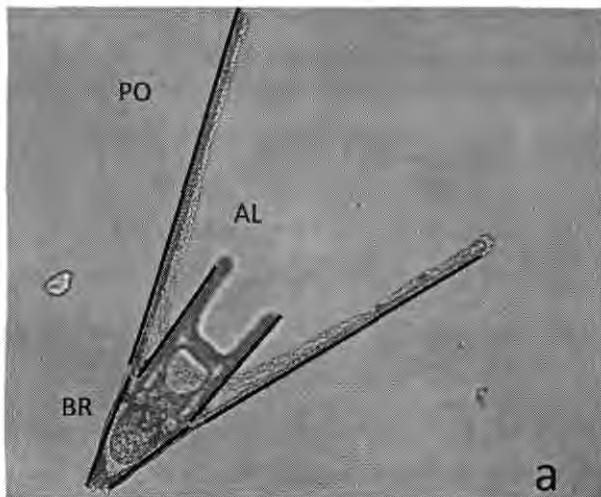


Figure 2. a. Skeletal measurements on *A. punctulata* larva, the left post oral arm (LPO), right post oral arm (RPO), left anterolateral arm (LAL), right anterolateral arm (RAL), left body rod (LBR), and right body rod (RBR). b. Body measurements on *A. punctulata* larva, body width (BW), body length from posterior tip to post oral tissue bridge (BL1), body length from posterior tip to anterolateral tissue bridge (BL2).

## Results

### *Rearing Effects of CO<sub>2</sub> on Larval Development*

Larvae raised under current (1x) CO<sub>2</sub>, had some skeletal characteristics that were longer than larvae raised under predicted future (2.5x) CO<sub>2</sub>. Average post oral (PO) arm length was significantly longer ( $F_{1,131} = 23.905$ ,  $P < 0.001$ ), under current levels (Figure 3, 8). Average body rod (BR) was longer at 1x CO<sub>2</sub> than at 2.5x ( $F_{1,126} = 18.410$ ,  $P = 0.036$ ; Figure 4, 8).

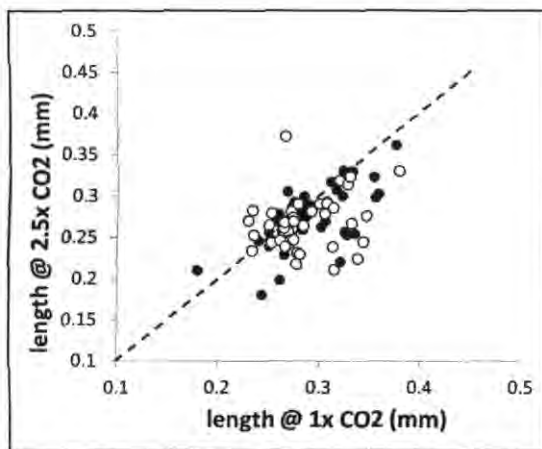


Figure 3. Effect of CO<sub>2</sub> on average PO arm length. Filled points are larvae fertilized under 1x CO<sub>2</sub>, and open points were fertilized under future 2.5x CO<sub>2</sub>.

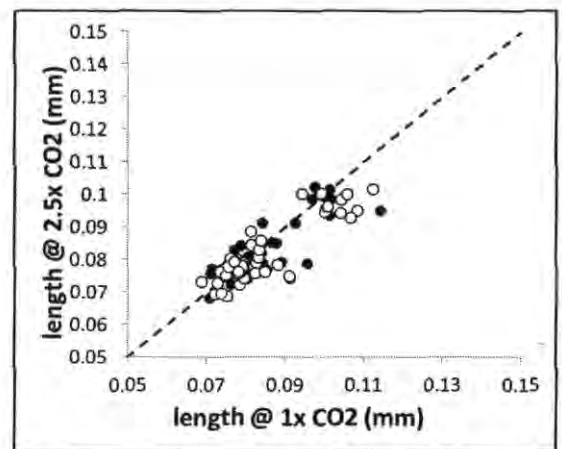


Figure 4. Effect of CO<sub>2</sub> on body rod length (BR). Symbols as in Figure 3.

### *Fertilization Effect of CO<sub>2</sub> on Larval Development*

Larvae fertilized under current (1x) CO<sub>2</sub>, had some skeletal and body tissue characteristics that were longer than larvae fertilized under predicted future (2.5x) CO<sub>2</sub>. Body rods were longer when fertilized under current 1x CO<sub>2</sub> levels ( $F_{1,127} = 4.509$ ,

$P = 0.036$ ; Figure 4, 8). Average anterolateral (AL) arm length was significantly ( $F_{1,143} = 4.075$ ,  $P < 0.001$ ) longer under current levels (Figure 5, 8). Body length (from the posterior tip to the postoral tissue bridge) was longer when fertilized under current 1x  $\text{CO}_2$  levels ( $F_{1,126} = 10.374$ ,  $P = 0.036$ ; Figure 6, 8).

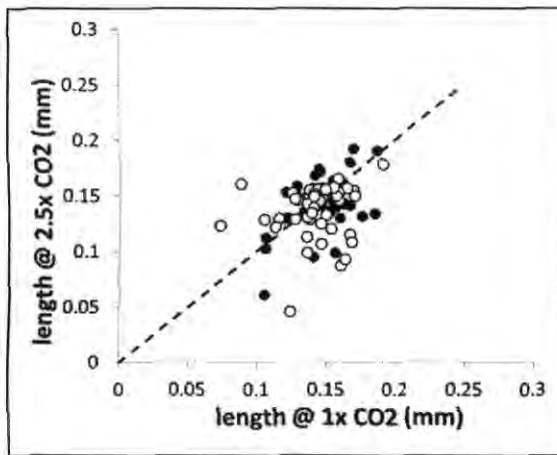


Figure 5. Effect of  $\text{CO}_2$  on average anterolateral arm (AL) length. Symbols as in Figure 3.

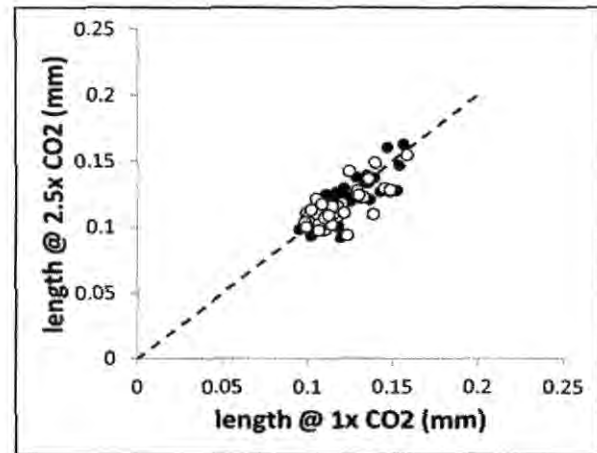
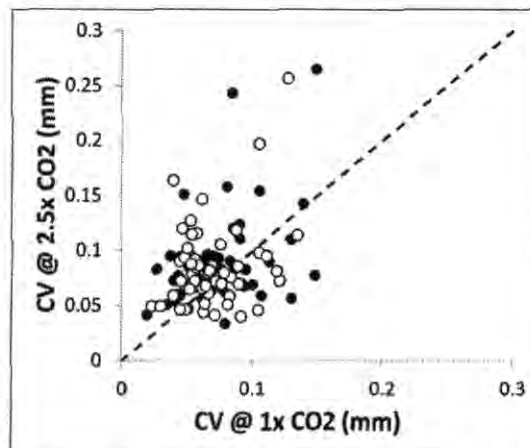


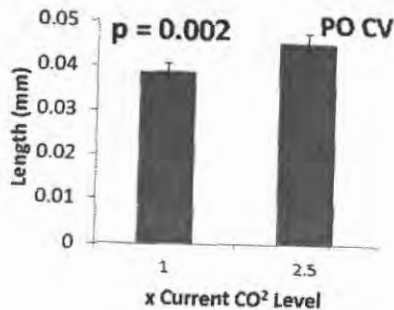
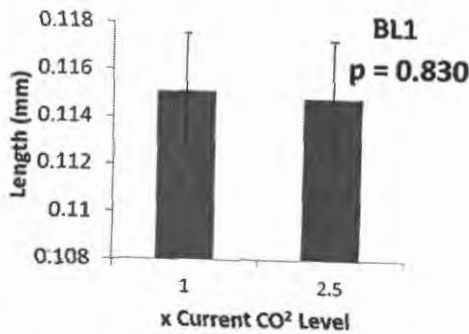
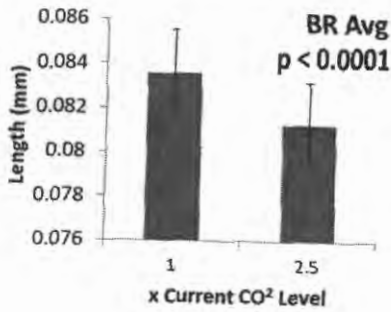
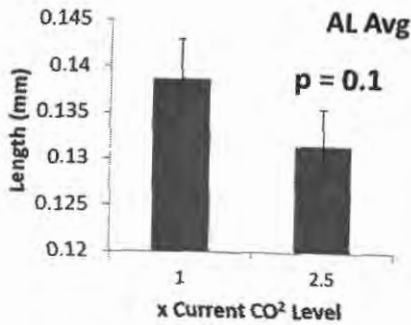
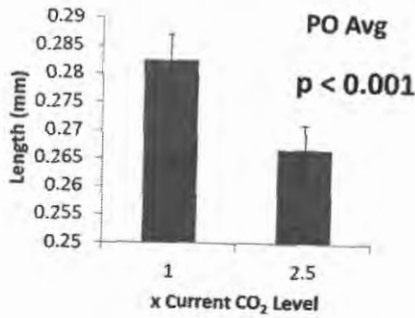
Figure 6. Effect of  $\text{CO}_2$  on body length (BL1). Symbols as in Figure 3.

#### *Effects of $\text{CO}_2$ on the Coefficient of Variation*

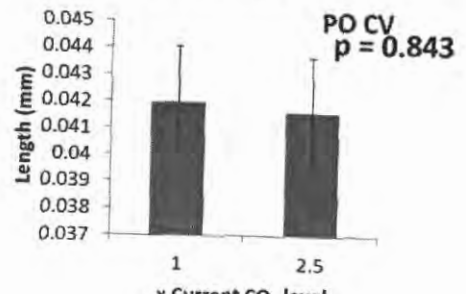
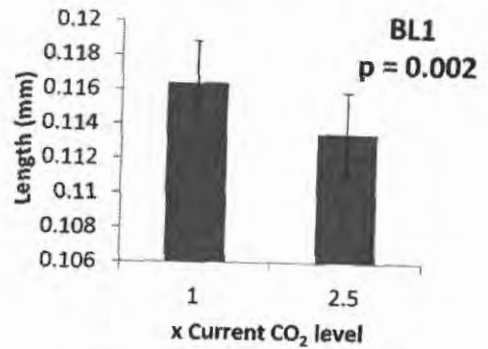
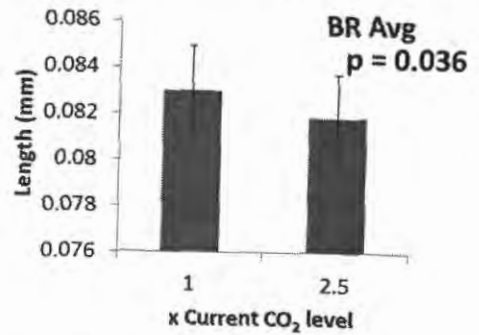
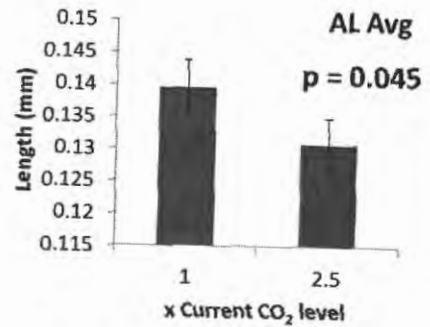
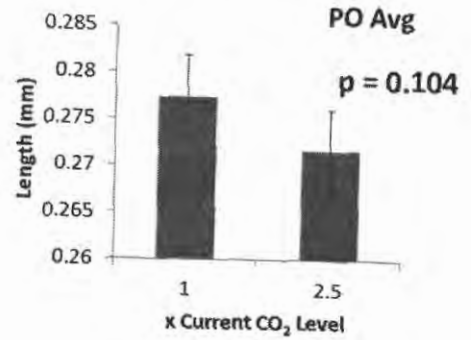
We also tested for asymmetry of several characters. For example, larvae raised under 2.5x  $\text{CO}_2$  had a significantly higher coefficient of variation (CV) of the left PO and right PO arms. PO symmetry was significantly more variable when reared at 2.5x  $\text{CO}_2$  ( $F_{1,143}=9.954$ ,  $P=0.002$ ; Figure 7, 8)



# Rearing



# Fertilization





### *Genetic Variation for Resistance to CO<sub>2</sub>*

We detected significant genetic variation on the size of certain characters but no evidence of genetic variation on the response to the change in rearing CO<sub>2</sub> (Table 10 a, b). Analyzing the effect of the genetic variation on size in response to CO<sub>2</sub>, male identity (representing additive genetic variation) had a significant effect on the two measures of body size. The interaction between male and female identity (representing non-additive genetic variation) was significant for the two body size characters and PO length. There was no significance of female identity on any character.

Table 10. Significance in the effect of Genetic Variation on the Size of Larvae (a) and Response to CO<sub>2</sub>

a.	Arm		Body		CV
	PO Length	AL Length	Rod Length	Length	PO
<b>Male</b>	-----	0.488	0.009	0.021	0.525
<b>Female</b>	0.073	0.224	-----	0.808	0.382
<b>Male X Female</b>	.026	-----	0.001	0.012	0.700

b.	Arm		Body	
	PO Length	AL Length	Rod Length	Length
<b>Male</b>	0.428	0.198	-----	0.420.
<b>Female</b>	-----	-----	-----	-----
<b>Male X Female</b>	0.733	0.373	-----	-----



## Discussion

We found evidence that morphological features of the larvae of the sea urchin *Arbacia punctulata* are affected by exposure to increased CO<sub>2</sub> during larval growth. For example, post oral arm size and body rod size were significantly smaller when reared under 2.5x versus 1x CO<sub>2</sub>. These results are consistent with other studies indicating that the growth of larvae, post oral arms in particular, are affected by a lower pH, or an increase in CO<sub>2</sub> levels (Clark et al. 2009, Brennard et al. 2010 ).

Larval skeleton size can significantly impact larval growth and survival. Shorter skeletal elementals like larval arms can be detrimental in survival, in that the arms of echinoderm larvae are necessary for locomotion, feeding, and protection from predators (Allen, 2008, Soars et al. 2009). It has also been seen that larvae that are smaller have reduced feeding capability because larval arms contain ciliated bands that allow food to travel downwards toward the mouth (Hart 1995). Larvae that are smaller and have a longer planktonic life stage will also likely have decreased survival and recruitment, because they are more susceptible to predation (Lamare et al. 2009).

Although we expected an effect of rearing conditions on larval growth, surprisingly we also found an effect in several cases of CO<sub>2</sub> conditions at fertilization on subsequent larval growth. Similar to the effects of rearing conditions anterolateral arms and body rods were smaller when eggs were fertilized at 2.5x CO<sub>2</sub>. However, unlike rearing conditions, fertilization conditions had a significant effect on the body length of larvae, as larvae fertilized at 2.5x current CO<sub>2</sub> had smaller body length than larvae raised under 1x CO<sub>2</sub>. We propose two hypotheses for how fertilization CO<sub>2</sub> conditions could

have this effect on larvae size: (1) fertilization conditions create selection on genes that will later affect later expression of traits, or (2) fertilization conditions have a plastic effect on trait expression later in development. We did not, however, detect any significant interactions between fertilization and rearing conditions, which would have been consistent with an adaptive response to CO<sub>2</sub>; in all cases, those fertilized under elevated CO<sub>2</sub> had slower growth.

In addition to an increased CO<sub>2</sub> level having an effect on the length of the larvae, increased CO<sub>2</sub> also affected the symmetry of certain characters. For instance, the difference in length between left and right post oral arms was significantly greater when reared at 2.5x current CO<sub>2</sub>. This evidence supports a greater deviation from symmetry when reared under more extreme CO<sub>2</sub> conditions. These results are consistent with studies showing that certain traits that are normally bilaterally symmetrical in other organisms, like bristle number in adult *D. melanogaster* or molars of rats, will show fluctuating asymmetry when under genetic or environmental stress, according to Parsons 1992.

We analyzed genetic variation in traits by considering both additive genetic characters as reflected by male identity, combination of potentially additive variation and non-additive variation (due to maternal effects) as reflected by female identity, and non-additive variation as reflected by a male-female interaction. We observed significant genetic variation for body length and body rod length, suggesting that these characters respond to selection. Male-Female interaction had a significant effect on the body length, body rod length, and post oral arm length of larvae, meaning that the compatibility of male-female pairs may play a role in larval growth. However, because the genetic

variation represented by these interactions will not be passed down to future offspring they cannot be under selection and cannot therefore influence how urchins will evolve.

Although we found significant genetic variation in larval size, there was no significant genetic variation detected for the response of larval growth to CO<sub>2</sub>. This is contradictory to some other studies which displayed that some sea urchin species do have the genetic variation to adapt to future ocean acidification conditions (Kelly et al. 2013, Pespeni et al. 2013). However, using a different sea urchin, the purple sea urchin (*Strongylocentrotus purpuratus*), these studies used animals from different collection sites for examining genetic variation. In contrast, we tested individuals from a single population, suggesting that individual populations may harbor less genetic variation for a future response to CO<sub>2</sub>. Larger studies may be necessary to detect such within-population variation.

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